

EFFECTIVENESS OF HALOGEN-BASED DISINFECTANTS AGAINST *Acinetobacter baumannii*: WOUND CARE AND ENVIRONMENTAL DECONTAMINATION

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ABSTRACT

Historically, *Acinetobacter baumannii* has not been considered an important human pathogen. However, a surprisingly high number of multidrug-resistant *A. baumannii* war wound, bloodstream and osteomyelitis infections are occurring in American soldiers injured in Iraq/Kuwait (Operation Iraqi Freedom) and in Afghanistan (Operation Enduring Freedom). Thermal projectiles, (e.g., shrapnel, projectile injuries, or traumatic blast) carry the bacterium directly into combat wounds. Because of its resistance to multiple antibiotics, *A. baumannii* is emerging as an important pathogen among combat casualty victims and has become a significant clinical disease. With the number of *A. baumannii* infections rapidly rising, added to *A. baumannii*'s well-established resistance to multiple antibiotics, underscores the relevance for identifying the most excellent disinfectants for reducing *A. baumannii* populations in combat wounds and combat health care settings.

We compared the bacteriostatic and bactericidal activities of five commercially available U.S. halogen-based disinfectants against *A. baumannii*, along with a standard *E. coli* comparator, in a novel bacterial culture system that incorporated a three log range of organic growth media concentrations. We report the highest dilutions of stock disinfectant able to inhibit replication or kill the bacteria, denoted as the maximum inhibitory dilution (MID) and maximum bactericidal dilution (MBD), respectively.

For Maximum Inhibitory Dilutions (MID), Stabilized Chlorine Dioxide (SCD) had the highest MID of the five disinfectants tested. BETADINE was nearly equivalent to SCD. SCD and BETADINE had the highest MID values against *A. baumannii*. SCD was superior against *E. coli* ($p < 0.001$) compared to UltraChlorox, Clidox-S, BioClenz, and BETADINE, and showed superior MID activity at high organic concentrations. For Maximum Bactericidal Dilution (MBD), BETADINE was superior against *A. baumannii*, as well as *E. coli* ($p < 0.001$). Overall, the least effective disinfectant against *A. baumannii* (and *E. coli*) was UltraChlorox. Among the five disinfectants contrasted,

SCD and BETADINE, were superior for the reduction of *A. baumannii* in samples contaminated with organic material. Chlorine dioxide disinfectants may provide significant bactericidal effects against a range of bacteria to include *A. baumannii* contaminated soldiers. In addition, they may provide a superior disinfecting capability in the healthcare environment, preventing the spread of this and other bacteria.

1. INTRODUCTION

Acinetobacter baumannii is a pleomorphic aerobic gram-negative bacillus that is widely distributed in nature and causes a wide spectrum of nosocomial infections. It has a capacity for long-term survival (up to several months) on most environmental and dry surfaces. While some discussion continues on the source of *A. baumannii* contamination of war wounds, its prevalence continues to increase. *A. baumannii* has been identified in environmental sampling at military treatment facilities along the Tigris River in Iraq and in Kuwait City, Kuwait. Davis, et al., 2005, evaluating *Acinetobacter* extremity infections in soldiers, reported the increased prevalence of multidrug-resistant *Acinetobacter* species war wound infections and osteomyelitis during the 2003-2005 military operations in Iraq. They initially suspected that colonized soldiers themselves were the reservoir for multidrug-resistant *Acinetobacter*, and that this colonization came from the environment. They based this hypothesis on the fact that these organisms are ubiquitous in the environment, and thus the inoculation of these war wounds during injury seemed plausible, and *Acinetobacter* species had previously been described as common pathogens in war wounds. However, these infections are apparently similar to reported nosocomial multidrug-resistant *Acinetobacter* infections. Military medical staff recognize that the numbers of *Acinetobacter* infections increase in battlefield situations because of the types of traumatic injuries service-members suffer.

The investigations of Dakin and collaborators (1916, et seq.), and the introduction of the solution bearing his name, for the treatment of surgical wounds during World War I, marked the beginning of the extensive use of chlorine antiseptics, in this case a 0.5%

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sodium hypochlorite solution. The standard care for the type of open and infected combat casualty wounds seen today includes vigorous and complete early irrigation and debridement. The wounds are packed with saline soaked Kling or fine mesh gauze and left alone for four days. If the wound does not appear clean at four days, it is debrided, irrigated and packed (sometimes with Dakin's solution, or one of the surgeon's choice) and the wound left alone for another four days. As antiseptics, chlorine containing compounds have largely been replaced by agents that are less irritating. Chlorine dioxide containing solutions may represent effective non-cytotoxic alternatives for wound care where organisms such as *A. baumannii* are present, and provide superior disinfection of the wound. However, chlorine containing compounds are used extensively as sanitizing agents, with elemental chlorine being one of the most potent germicidal agents. The concentration of chlorine necessary to kill most organisms, in 15 to 30 seconds, varies between 0.10 and 0.25 ppm.

As the increasing prevalence of antibiotic resistance continues to restrict treatment options, the role of wound care, infection control, and environmental decontamination become more important. Halogen-based commercial disinfectants have not been studied against *A. baumannii*. UltraChlorox and BETADINE Scrub, are commonly used as disinfection agents, effective in inhibiting the replication of (or killing of) a wide range bacteria, yeast and viruses. Chlorine dioxide disinfectants are also effective against a wide range of microbes, but they have not been extensively studied.

Comparative disinfectant studies often suffer from not including organic material in the test solution, a most important omission considering the military healthcare environment and the nature of combat wounds. A highly proteinaceous environment, with buffering capacity and agent-neutralizing potential, "stacks the deck" against disinfectant efficacy. Long chain amphipathic lipids and protein-binding interaction(s), e.g., albumins, sequester the disinfectant, disabling antimicrobial activity. Organics facilitate a nutritive-base for rapid recovery of the reduced bacterial population. Disinfectants such as bleach and quaternary ammonium compounds are inactivated under heavy soil load. In the assessment of the ability of disinfectants to provide a bacteriostatic or bactericidal effect, heavy organic material is essential in today's environment.

Here we compared the bacteriostatic and bactericidal activities of five commercially available U.S. halogen-based disinfectants against *A. baumannii*, along with a standard *E. coli* comparator, in a novel bacterial culture system that incorporated a three log range of organic growth media concentrations. Values are reported as the highest dilutions of stock disinfectant

able to inhibit replication or kill the bacteria, denoted as the maximum inhibitory dilution (MID) and maximum bactericidal dilution (MBD), respectively.

2. MATERIALS AND METHODS

We tested the antibacterial properties of five halogen-containing disinfectants: UltraChlorox Germicidal Bleach (sodium hypochlorite 6.15%, Chlorox Co, Oakland, CA), BETADINE Surgical Scrub, Povidone-iodine (7.5%, Purdue Frederick Co, Stamford, CT), Clidox-S® (Pharmaceutical Research Labs, Naugatuck, CT), prepared immediately before use by adding, one part Activator to one part Base, BioClenz™ (Frontier Pharmacy, Melville, NY), prepared immediately before use by adding, one part Activator to one part Base, and Stabilized Chlorine Dioxide concentrate (SCD, 5%, Chlorway Products, Phoenix, AZ). SCD is chlorine dioxide "stabilized" in an aqueous buffered solution (no premixing required). Normal saline was used as a control in all experiments.

In all experiments, the nomenclatural "type" strain for species *Acinetobacter baumannii* was used. The American Type Culture Collection (ATCC, Manassas, VA) has designated the Bouvet and Grimont strain, ATCC 19606, for *Acinetobacter baumannii* (*Int. J. Syst. Bacteriol.* 36, 228, 1986). It was originally isolated from human urine. ATCC 19606 was selected for use by Heinemann et al., 2000 for comparative activities of fluoroquinolones against *A. baumannii*. As a comparator microbe, we used *Escherichia coli* (type strain ATCC 11775) in all experiments. Strain ATCC 11775 was isolated from human urine by Kaufmann in 1941; its antigenic properties are: serovar O1:K1:H7 (*Int. Bull. Bacteriol. Nomencl. Taxon.* 13:35-36, 1963).

2.1 Experimental Conditions

Disinfectants were compared for bacteriostatic and bactericidal activities at an identical range of dilutions of stock solutions (undilute to 1:1024). Undilute (100 µL) aliquots of each disinfectant were added to individual wells of 96-well U-bottomed sterile polystyrene microtitration plates (Costar, Cambridge, MA), and then serially diluted in saline (100 µL), using two-fold dilutions.

Brain Heart Infusion (BHI, Difco, Detroit, MI), a common growth media in microbiology, was used in this study as the "organic", to interfere with the antibacterial activity of the disinfectants. BHI, made of infusions of calf brain and beef heart, containing proteose peptone and dextrose, well fits the definition of an "organic", as a carbon and hydrogen nutritive source able to promote bacterial binary fission. BHI (100%

strength) was prepared according to the manufacturer's directions (37 g/L), then autoclaved. It was diluted with sterile deionized water to create organic strengths of 10% and 1%. BHI (100 μ L aliquots) was added to rows of microtiter plates containing diluted disinfectant, then wells were mixed on a rotating platform mixer at room temperature for two minutes, before the addition of bacteria.

2.2 MID and MBD Determinations

Bacteria were grown in Tryptic Soy Broth (TSB, Difco) for three hours to obtain log-phase growth, for optimal microbial viability, then adjusted to an A_{540} turbidometric value of 0.1 (ca 10^7 colony-forming-units per ml, cfu/ml). Bacteria were diluted in cold (4° C) phosphate buffered saline (PBS, 50 mM, pH 7.4, 1:500 final dilution) to dilute the organic material in the bacterial-culture solution, and stored on ice to minimize change in bacterial density prior to use. Aliquots (50 μ L) of bacteria were added to all wells of the 96-well microtiter plate, except for the last row to assess for potential bacterial contamination.

Following incubation on a rotating mixer for two minutes, 2 μ L aliquots from each well of Plate A were transferred, using a sterile 96-pronged inoculator handheld device, to corresponding and identically-located wells of a second microtiter plate (Plate B). All wells of Plate B contained Tryptic Soy Broth (100 μ L) as growth medium. Both plates were placed at 37° C in 5% CO₂ and cultured overnight. The next morning, U-bottomed wells were examined for the presence/absence of a formed bacterial pellet, and/or growth-related turbidity. Maximum Inhibitory Dilution (MID) was defined from Plate A, as the highest dilution that inhibited bacterial cell division, not yielding growth or pellet, and having a bacteriostatic effect. The Maximum Bactericidal Dilution (MBD) was defined from Plate B, as the highest dilution that yielded an eradication of viable bacteria (nonviability following transfer to fresh media), or a bactericidal effect. At least three replicate determinations were done for each variable under study.

2.3 Data Analysis

Mean dilutions were contrasted by Two Way ANOVA, SigmaStat version 3.0 software (SPSS Inc., Chicago, IL), followed by a pairwise multiple comparisons test (Tukey's). A "p" value ≤ 0.05 was considered significant.

3. RESULTS

3.1 Maximum Inhibitory Dilution

Over all organic concentrations under study, and against *A. baumannii*, MID values differed among disinfectants ($p < 0.001$). Pairwise comparisons showed SCD to possess high MID values. SCD dilution was higher than UltraChlorox ($p < 0.001$) and Clidox-S® ($p = 0.002$), and comparable to BETADINE ($p = 0.194$) or BioClenz™ ($p = 0.27$) (Figure 1).

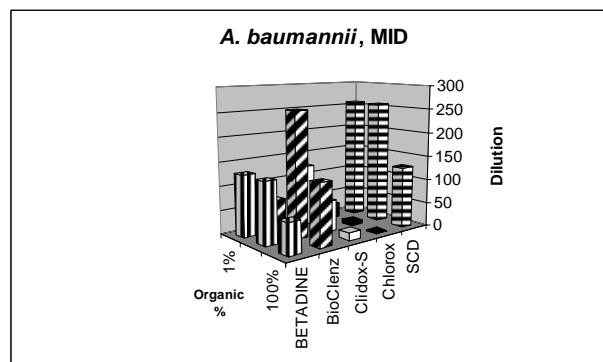


Figure 1. The Maximum Inhibitory Dilution of disinfectants having a bacteriostatic effect on *A. baumannii* in the presence of increasing amounts of organic material. SCD and BETADINE possessed a superior bacteriostatic effect as dilution of the disinfectant increased, and as the organic material increased ($p \leq 0.001$).

Against *E. coli*, MID values also differed among disinfectants ($p < 0.001$). Again, SCD showed high MID values (Figure 2). SCD dilution was higher than UltraChlorox ($p < 0.001$), Clidox-S® ($p < 0.001$), BioClenz™ ($p = 0.007$), and BETADINE ($p = 0.015$). BETADINE's dilution was higher than UltraChlorox ($p = 0.002$), but not Clidox-S® ($p = 0.69$), or BioClenz™ ($p = 1.0$); BioClenz™ dilution was higher than UltraChlorox ($p = 0.003$).

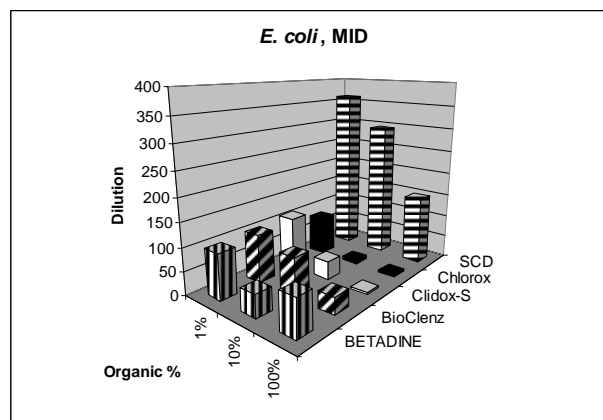


Figure 2. The Maximum Inhibitory Dilution of disinfectants having a bacteriostatic effect on *E. coli* in the presence of increasing amounts of organic material. SCD possessed superior bacteriostatic effect as the

dilution of the disinfectant increased, and as the organic material increased ($p \leq 0.001$).

3.2 Maximum Bactericidal Dilution

Over all organic concentrations under study, and against *A. baumannii*, MBD values differed among disinfectants ($p < 0.001$) and levels of organic material ($p < 0.011$). But, for bactericidal activity, BETADINE showed high MBD values. Derived least square means (± 1 SE) for Betadine were higher than UltraChlorox ($p < 0.001$), but marginally higher than the other disinfectants. SCD and Clidox-S® showed better bactericidal activity vs. UltraChlorox ($p \leq 0.002$), but were not different from each other ($p = 0.25$). BioClenz™ was also higher than UltraChlorox ($p = 0.025$) (Figure 3).

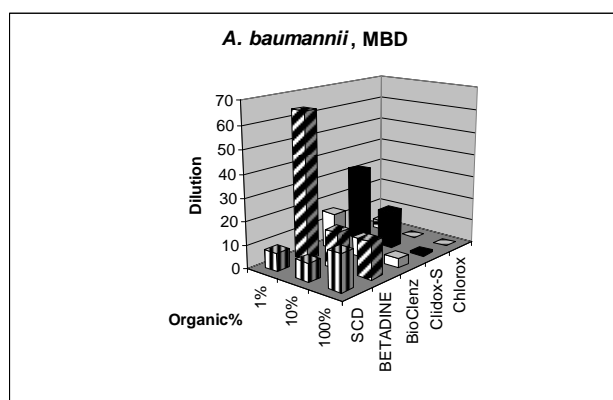


Figure 3. The Maximum Bactericidal Dilution of disinfectants capable of eradicating viable *A. baumannii* in the presence of increasing amounts of organic material. BETADINE exhibited superior activity at low organic material ($p \leq 0.001$).

Against *E. coli*, MBD values differed widely among disinfectants ($p < 0.001$) and levels of organic material ($p < 0.001$). Again, BETADINE showed the highest MBD values. Pairwise multiple comparisons showed BETADINE to be higher than SCD, BioClenz™, Clidox-S® and UltraChlorox (all, $p < 0.001$). Clidox-S® was higher than UltraChlorox ($p < 0.001$), but not SCD ($p = 0.12$) or BioClenz™ ($p = 0.178$). Among tested disinfectants UltraChlorox yielded low bactericidal activity against *E. coli* (Figure 4).

4. DISCUSSION

Among the disinfectants under study, SCD had comparable or higher antibacterial activity vs. both *E. coli* and *A. baumannii*, identifiable in the presence of high amounts of organic material. Stabilized chlorine dioxide is a powerful oxidizing agent and superior to sodium hypochlorite. It is used in Europe to treat

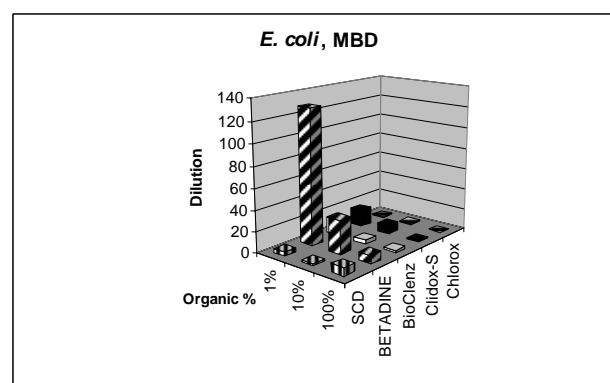


Figure 4. The Maximum Bactericidal Dilution of disinfectants capable of eradicating viable *E. coli* in the presence of increasing amounts of organic material. BETADINE exhibited superior activity at low organic material ($p \leq 0.001$).

drinking water because it is nontoxic and does not form carcinogenic by-products (like sodium hypochlorite). Disinfectants containing chlorine dioxide display a high antibacterial activity in the presence of high amounts of organic material, have the ability to break down phenolic compounds, and lacks reactivity with ammonia (Rutala and Weber, 1997). This makes them ideally suited for use in all military health care environments. Their non-toxicity may also make them valuable in early wound care in irrigation solutions, as wound cleansing agents, or as part of a wound packing agent in a bandage suitable for field use. Chlorine dioxide containing solutions may provide important infection control during early treatment in far forward-deployed combat support hospitals and medical treatment facilities as an easy to use disinfectant, as decontamination of healthcare and transport settings throughout the MEDEVAC chain and environmental contamination control.

For Maximum Inhibitory Dilutions (MID), Stabilized Chlorine Dioxide (SCD) had the highest MID of the five disinfectants tested, and BETADINE was nearly equivalent to SCD, and both had the highest MID values against *A. baumannii*. SCD was superior against *E. coli* ($p < 0.001$) compared to UltraChlorox, Clidox-S®, BioClenz™, and BETADINE, and showed superior MID activity at high organic concentrations. For Maximum Bactericidal Dilution (MBD), BETADINE was superior against *A. baumannii*, as well as *E. coli* ($p < 0.001$). Overall, the least effective disinfectant against *A. baumannii* (and *E. coli*) was UltraChlorox. Among the five disinfectants contrasted, SCD and BETADINE, were superior for the reduction of *A. baumannii* in samples contaminated with organic material.

Among the five halogen-based disinfectants contrasted in this study, two, Stabilized Chlorine

Dioxide, and BETADINE Surgical Scrub, showed excellent antibacterial activity against *A. baumannii*. SCD showed a bacteriostatic effect, while BETADINE Scrub had a bactericidal effect.

Russell, 1998 was the first to recognize the inappropriate use of Minimum Inhibitory Concentrations (MICs) when antiseptics and disinfectants are being considered. The resistance of a bacteria to an antibiotic is generally clear and concise, and the MIC can be readily obtained. The lethal effects of antiseptics and disinfectants are considerably more important, and more complicated by their very nature. We used dilution-based comparisons of commercial disinfectants in this study, instead of concentration-based comparisons. A most relevant corollary is, as Russell pointed out, antibiotics and minimum inhibitory concentrations (MIC). The MIC is the lowest active agent concentration causing a demonstrable antibacterial effect. MIC testing requires knowledge of the active agent's concentration, and implicit to the comparison, is the assumption that single agents are compared. For commercially-available disinfectants, proprietary alterations, e.g., second-agent additions and in-solution activation of agents, make comparisons much more difficult, and may impede comparisons. A good example of this is Lysol spray, containing 79% ethyl alcohol, a base ingredient for many disinfectants, and only 0.1% orthophenylenol. Alcohol, as a second-agent addition, is an excellent disinfecting agent, but must be left in contact with the item to be disinfected for twenty minutes to be effective. To overcome this, we propose defining maximum inhibitory dilution (MID), as the highest dilution of undilute disinfectant, able to inhibit bacterial cell division (a bacteriostatic effect). The maximum bactericidal dilution (MBD) was then defined as the highest dilution of commercially available disinfectant that eradicates all viable bacteria. Russell recognized that although MICs are appropriate for antibiotic sensitivity testing, it is not suitable for testing the biocidal activity of antiseptics and disinfectants, and we have proposed a proper means to assess that antiseptic and disinfectant activity, MIDs and MBDs.

Martro et al., 2003, tested the susceptibility of nine clinical isolates of *A. baumannii* against several European commercial antiseptics and disinfectants. Sulphate, quaternary ammonia and phenolic agents were among the types tested *in vitro*. None contained chlorine dioxide and the inhibitory effect of organic matter was not assessed. All *A. baumannii* strains were equally sensitive to the use-dilution of the two disinfectants and the antiseptics tested, showing good bactericidal activity. Their results showed no evidence of a correlation between antibiotic and biocide resistance, or development of resistance over time, confirming the work of Barry et al., 1999, who concluded that antibiotic

resistance had no effect on sensitivity to either chlorhexidine gluconate or povidone-iodine when testing *Acinetobacter* and other species. Barry et al., 1999 also assessed the disinfection or antisepsis effect of chlorhexidine gluconate and povidone iodine against 50 *Acinetobacter* sp., determining the MIC of both at various dilutions. They concluded both inhibited at concentrations well below those that are normally used.

Jennane, et al, 1995, tested seventy-nine hospital strains of *Acinetobacter baumannii* for sensitivity to the disinfectants routinely used in their hospital. After five minutes of contact with the bacteria, disinfectants containing aldehyde were 98% effective,, containing phenol were 98% effective and containing amphotere were 93% effective. All were 100% effective with a ten minute contact time. They tested two halogen containing disinfectants and found them ineffective at five minutes. They did not test dilutions of the disinfectants, or their ability to kill *A. baumannii* in the presence of an organic material.

Bactericidal agents lethally reduce bacterial populations. However, their mode of action may simultaneously cause host toxicity. BETADINE Scrub is a broad-spectrum skin cleanser for pre- and postoperative use as a microbiocide. It is for external use only and toxic if swallowed. While BETADINE may be an effective skin disinfectant for *A. baumannii*, it might not be well accepted by health care providers. While bleach has broad antimicrobial activity, it also has similar disadvantages of BETADINE, of irritation to mucous membranes, causing mild irritation to frank necrosis of tissues when used at high concentrations, and decreased efficacy in the presence of organic material (Rutala and Weber, 1997). Nontoxic chlorine dioxide containing products may be ideal for hand hygiene to aid in the control of the spread of *A. baumannii* by health care providers, for decontamination of the healthcare environment, and for wound care itself. Handwashing is the single most important procedure for preventing hospital-acquired infections. Cardoso et al., 1999, tested the effectiveness of four hand-cleansing agents for their ability to remove *A. baumannii* from artificially contaminated hands. Although all agents were found to be effective, 70% ethyl alcohol and 10% povidone-iodine removed significantly more bacteria from heavily contaminated hands than plain soap or 4% chlorhexidine gluconate.

CONCLUSION

Chlorine dioxide containing agents may offer a new non-cytotoxic alternative to existing disinfectants in the treatment of wounds and in environmental decontamination. As the number of *A. baumannii*

infections increase, and their resistance to multiple antimicrobial agents increase, the importance of infection control during treatment in a combat military medical facility, and the health-care environment itself, encourages us to evaluate new treatment and decontamination options. The disinfectants used should be effective against the expected organisms. Additional investigations are underway to evaluate their usefulness as both a disinfectant in the healthcare setting, and to determine their usefulness in wound care management.

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